Poster presentations

SESSION I: Novel opioid peptide agonists and antagonists

PS I – 1

Design and synthesis of opioid peptide analogues and mimetics

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Background and Aim: Native opioid peptides possess enormous promise for the treatment of pain. However, despite this potential, such peptides have seen no use as clinically viable drugs for their low stability against proteolysis, resulting in a short duration of *in vivo* activity, and for their poor ability to cross the blood-brain barrier. One way to overcome these disadvantages is the use of modified peptides, the so called peptidomimetics [Gentilucci et al., 2010].
Methods: Starting from the structure of Endomorphin-1 (Tyr-Pro-Trp-PheNH2), the endogenous ligand of μ-opioid receptor (MOR), we prepared modified peptides and peptidomimetics by introduction of unnatural amino acids, halogenation of the Trp, introduction of Pro-analogues, lipidization, and size reduction. The syntheses of (S)- or (R)-halo-tryphtophans and other Trp-analogues have been performed by tandem 1,4-addition and stereoselective enolate protonation of DHA-containing peptides. Otherwise, substituted tryphtophans have been prepared in optically pure form by enzymatic resolution. Oxazolidin-2-one4-carboxylate has been introduced in position 2 of endomorphin-1 as a proline mimetic, to give a trans conformation of the Tyr1-Xaa2 peptide bond. Lipidization and a rationale search for focused structure modifications lead to the endomorphin mimetic N-Ac-D-Trp-Phe-Gly-NH2 and halo-substituted derivatives.
Results: The modified peptides and mimetics have been bio-assayed *in vitro* by binding experiments, to determine the affinity for MORs, and *in vivo*, to verify the analgesic activity; the bioactivity has been rationalized by molecular docking computations.
Conclusion: The new endomorphin analogues and mimetics described here constitute promising leads for the development of potentially useful, lipophilic analgesics.

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PS I – 2

Novel opioid peptides from genomic data banks: phylogenetic and bioactivity studies

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Background and Aim: Endogenous opioid peptides are processed from large molecular mass precursor polypeptides encoded by distinct genes. Proopiomelanocortin (POMC), Proenkephalin (PENK), Prodynorphin (PDYN) and Pronociceptin (PNOC) are the four ubiquitous precursors, and their respective genes are located in different chromosomes. In lower vertebrates, mainly in fish species, additional copies of such precursor propeptide genes exist. Our aim was to collect and biochemically characterize novel opioid sequences identified by data mining.
Methods and Results: Our systematic database survey reveals that individual peptides identified in genomic or cDNA databases of various species exhibit remarkable polymorphism due to mutational changes. However, the purifying selection during evolution well restored those sequence motifs which are responsible for the biological activites. Variable regions can therefore be found mainly in the C-termini of the opioid oligopeptides. The YGGF tetrapeptide segment is the most conserved region even in the case of a number of non-mammalian nociceptin, indicating that the ancestral forms of nociceptin contained tyrosine in the first position. Some bioactivity studies, including radioreceptor binding and G-protein activation, with the chemically synthesized forms of the newly identified peptides will be presented.
Conclusion: The entire set of opioid peptides from different taxa constitutes natural- (NPL) or phylogenetic- (PPL) peptide libraries. NPL for endogenous opioids seems to be important for phylogenetic, comparative bioactivity and systems biology studies. The rapidly increasing content of NPLs displays amazing structural variegation, herewith it is a component of the chemical biodiversity.

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PS I – 3

A putative pharmacophore model of μ-opioid receptor activity

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Background and Aim: Many natural and synthetic peptides and peptidomimetics were shown previously to bind to the μ-opioid receptor (MOR) but there is no consensus about the structure responsible for biological activity. A putative pharmacophore model was established by our recent structure-activity study in which ten μ-opioid receptor ligands and their analogues, possessing different μ-opioid receptor affinity and selectivity were examined using molecular dynamics.
Results: Here we present the structural analysis of a small pool of endomorphin derivatives containing Dmt1 (2',6'-dimethyltyrosine), Achc2 (2-aminocyclohexanecarboxylic acid), pFPhe4 (para-fluorophenylalanine) or βMePhe4 (β-methyl-phenylalanine) substitutions. These μ-opioid ligands were shown to have similar or better pharmacological properties than those of the parent compounds. Our present NMR spectroscopic analysis and molecular modeling data

indicated that the solution structures of these high-affinity MOR ligands are in perfect agreement with our previously proposed bioactive structure model, in which the trans χ1 rotamer of Tyr1/Dmt1, the gauche conformer of Phe3 and the bent backbone structure were assumed to be responsible for the high μ-opioid activity.
Conclusions: Information obtained here could possibly resolve many contradictions in the past literature of opioid peptide structures and may serve as a guiding principle in the pre-screening of possible structural candidates in the design of novel μ-opioid agonists.

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PS I – 4

Cardiorespiratory effects of morphine and chimeric peptide in anaesthetized rats

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Background and Aim: The use of opioid analgesics is limited by a variety of side effects. AWL3106 – recently synthesized chimeric peptide comprising two pharmacophores with agonistic activity towards μ-opioid (dermorphin) and tachykinin NK1 (substance P) receptors presents 30 folds stronger analgesic activity than morphine. The aim of this study was to compare cardiorespiratory effects induced by AWL3106 and a model opioid analgesic – morphine.
Methods: We measured cardiorespiratory parameters in 16 anaesthetized rats, breathing spontaneously room air, subdue to an intravenous (*iv*) injection of 0.3 μmol/kg of either AWL3106 (n = 9) or morphine hydrochloride (n = 7).
Results: Bolus injection of AWL3106 evoked an apnoea of mean duration of 5.1 ± 0.7 s in all rats, followed by breathing of 19% reduced frequency (f) and augmented by 23% tidal volume (VT), which resulted in no change in minute ventilation. Morphine administered at the same dose, in five out of seven rats induced apnoea lasting on average 3.8 ± 1.1 s, which was not statistically different from that provoked by AWL3106. There was no effect on the frequency of breathing, but 17% transient decrease in VT resulted in short-lived decline in post-apnoeic ventilation. Both substances lowered mean arterial pressure (MAP). Morphine transiently reduced MAP by 24% at 15 s after drug challenge. Hypotension evoked by AWL3106 was biphasic and reached the lowest value immediately after injection at a rate of 63% and at 43% between the first and second minute, compared with control values.
Conclusion: This study showed that *iv* morphine induces respiratory depression due to the lack of compensatory mechanism. AWL3106 presents an additive effect on respiratory pattern which is beneficial to ventilation though strongly affects MAP.

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PS I – 5

Evaluation of constrained amino acids in the design of neurokinin 1 receptor- and bifunctional opioid – neurokinin 1 receptor ligands

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Background and Aim: Polypharmacology is generally possible in three ways: 1) drug cocktails, 2) multicomponent drugs and 3) multiple ligands. The first two methods show disadvantages like poor patient compliance and drug-drug interactions. Multiple ligands however, consisting of a single chemical entity that modulates multiple targets simultaneously, potentially overcome these drawbacks [Morphy et al., 2005; 2007].
Methods: Earlier research by Lipkowski and Hruby demonstrated that bifunctional peptides possessing both NK1R antagonist properties and opioid agonist potency show advantages over current analgesic drugs, such as a potent analgesic effect in both acute and neuropathic pain states, suppression of the development of tolerance and the presentation of antiallodynic and antihyperalgesic effects [Bonney et al., 2004; Hruby et al., 2009; Yamamoto et al., 2010].
Results and Conclusions: Different types of constrained aromatic amino acids were derivatized to prepare new neurokinin 1 receptor (NK1R) antagonists. Subsequent identification of the most potent antagonist allowed the preparation a chimeric structure possessing the desired dual opioid-NK1 activity (NK1 Ki = 0.5 nM, pA2 = 7.8; MOR Ki = 0.4 nM) Our latest efforts in the construction of this type of ligands will be presented.

PS I – 6

Opioid and anti-opioid peptides derived from plant proteins

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Background and Aim: We have found various types of opioid and anti-opioid peptides derived from plant proteins. Rubiscolin-6 (YPLDLF) has been isolated as a δ agonist from an enzymatic digest of spinach ribulose bisphosphate carboxylase/oxygenase (Rubisco), which is the most abundant protein on the earth existing ubiquitously in photosynthetic organisms. Although the antinociceptive activity of rubiscolin-6 was small, it exerted anxiolytic-like and memory-consolidating effects in the elevated plus-maze and the step-through type passive avoidance experiments, respectively. Interestingly, the σ1, $5HT_{1A}$ and D_1 receptors were involved in the anxiolytic effect, while the σ1, $5HT_{1A}$ and D_2 receptors were involved in the memory-consolidating effect, respectively, downstream the δ receptor.
Results: Rapakinin (RIY) has been isolated as a inhibitory peptide for the angiotensin I-converting enzyme. However, its antihypertensive effect was mainly due to the CCK_A receptor-dependent vasorelaxation. Rapakinin exerted an anti-opioid effect in the tail-pinch

test in the CCK_B receptor-dependent manner. It also showed a memory-consolidating effect in the CCK_B receptor-dependent manner. Rapakinin might stimulate CCK release downstream of an unidentified receptor since it showed no affinities for the CCK receptors.

Conclusions: It should be noted that these plant-derived peptides might have been on the earth far much earlier than the evolution of their receptors in animals.

PS I – 7

Design, synthesis and pharmacological evaluation of novel endomorphin analogues with multiple structural modifications

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Background and Aim: A major goal in opioid peptide research is the development of novel analgesics that could substitute morphine without its well-known side-effects of dependence, tolerance, respiratory depression and reward-seeking behavior [Keresztes et al., 2010]. Endomorphins (EM-1, YPWF-NH2, EM-2, YPFF-NH2) are high-affinity, μ-selective endogenous opioids which inhibit pain without some of the undesired side-effects of plant opiates [Zadina et al., 1997]. These properties render endmorphins (EMs) the object of continuous investigations and a promising target for the development of new opioid analgesics. Although EMs suffer from serious limitations, including short duration of action, lack of activity after oral administration, relative inability to cross the blood-brain barrier (BBB) into the central nervous system (CNS) and poor metabolic stability.

Results: Herein, we report on the synthesis and pharmacological evaluation of a small pool of endomorphin derivatives containing unnatural amino acids (Dmt1, Achc2, pFPhe4 and βMePhe4). The multiple structural modifications resulted in proteolytically stable and pharmacologically active analogues. Depending upon the chiralities of incorporated amino acid, analogues have exhibited moderate to high binding potencies, selectivities and efficacies.

Conclusions: In the present investigation we obtained the stable analogues with improved pharmacological properties.

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PS I – 8

Structural modification and biological evaluation of Dmt1-DALDA analogues

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Background and Aim: The highly charged tetrapeptide Dmt-DALDA (Dmt-D-Arg-Phe-Lys-NH2) has been previously identified as a potent μ-opioid receptor agonist1 and serves as a lead compound for the further development of novel therapeutic (peptidic) opioid analgesics [Shimoyama et al., 2008; Zhao et al., 2003]. The present work describes structural modifications of the peptide in order to determine the role of the charges, role of N-methylation, and role of conformation.

Methods: All prepared compounds have been tested for their *in vitro* affinity and activity (guinea pig ileum GPI and mouse vas deferens MVD assays), their *in vitro* permeability (caco-2 test) and *in vivo* tissue distribution, in- and efflux into and out of mouse brain.

Results: These experimental data indicate that: i) side-chain charges are not essential for *in vitro* activity, ii) the guanidine group of D-Arg2 is important for the blood-brain permeability iii) the conformational constraint of the Phe residue by the benzazepine ring results in highly potent compounds, but is not compatible with the Lys side chain, which can best be removed for high potency. A more detailed discussion of the obtained results will be presented.

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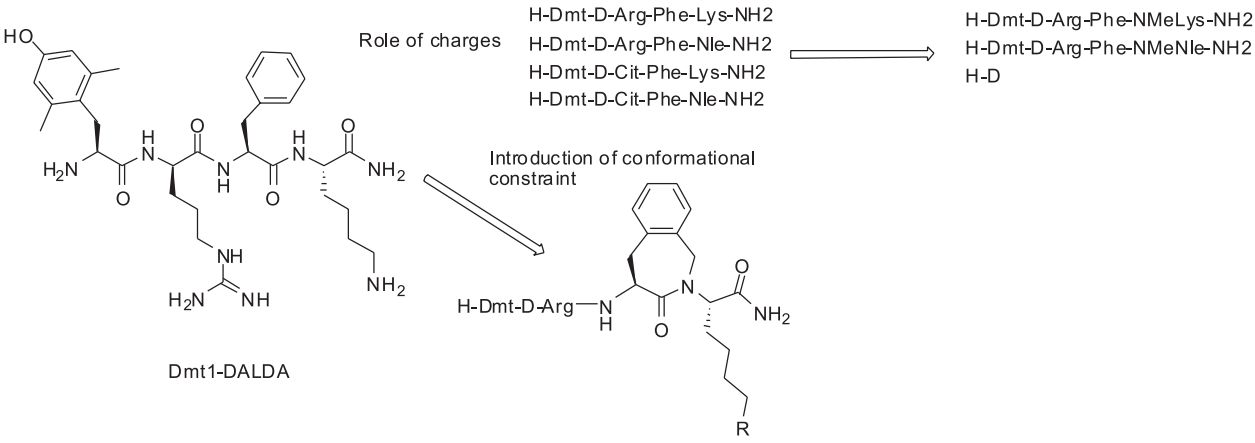

PS I – 9

Development of new opioid agonist tachykinin antagonist chimeric compounds

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Background and Aim: Our group proposed to develop new chimeric analgesics in which opioid pharmacophores are covalently hybridized with other types of pharmacophores that positively modulate effects of the opioid part. Synergistic enhancement of opioid analgesia and/or decrease of unwanted side-effects should result from such hybridization. It is generally accepted, that opioids and tachykinins are classified as functional antagonists. Therefore, hybridization of opioid agonist with tachykinin antagonist should result with very effective analgesics. Melanoma cancer cells overexpress tachykinin receptors. It has been already documented that tachykinin antagonists express antiproliferative properties of melanoma cells. Therefore chimeras of opioid agonists and tachykinin antagonists should express both analgesic and anticancer properties that make them ideal for cancer pain treatment. Series of new opioid agonist-tachykinin antagonists conjugates have been developed, synthesized and tested.
Methods: Series of new opioid agonist-tachykinin antagonists conjugates have been developed and synthesized. The affinities to opioid receptors μ and δ has been evaluated. The antiproliferative properties of new compounds has been evaluated *in vitro* in comparison to substance P antagonist aprepitant
Results: New compounds express high affinity to opioid receptors as well as antiproliferative properties of melanoma cancer cells *in vitro*.
Conclusion: New compounds are good candidates for further studies as analgesics for cancer pain treatment.

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PS I – 10

Synthesis and *in vitro* characterization of new salvinorin a analogs incorporating natural amino acids

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Background and Aim: A series of new derivatives of salvinorin A (SA), substituted at the C(2) position with natural non-aromatic and aromatic amino acids, was synthesized. The binding properties of SA analogs were characterized in the *in vitro* studies.
Methods: SA was isolated from S. divinorum, purified and converted to salvinorin B (SB) according to the methods established in our laboratory. New analogs were synthesized from SB and N-Fmoc-protected amino acids using N-methylmorpholine as a catalyst and TBTU as a coupling reagent, followed by deprotection with piperidine. For all the compounds spectral data were obtained. The κ-opioid receptor (KOR) and [^{35}S]GTPγS binding assays were conducted as described previously.
Results: The introduction of N-Fmoc-protected amino acids resulted in loss of affinity at KOR. The removal of Fmoc from Val-substituted analog in-

creased its affinity at KOR binding sites, making it almost equipotent with the parent compound, SA (K_i values of 42.0 ± 2.05 and 0.75 ± 0.62 nM for Val-SA *vs*. SA, respectively). As shown in the [^{35}S]GTPγS binding assays, Val-SA was a full agonist at KOR.

Conclusion: Using a novel approach, a series of new amino acid derivatives of SA was synthesized. One of the obtained analogs, Val-SA, displayed high affinity and full agonist activity at KOR, comparable to that of the parent compound.

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SESSION II: Opioid agonist specific signaling and regulation

PS II – 1

Opioids and the apoptotic pathway in MCF-7 breast cancer cell line

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Background and Aim: Apoptosis is an active process of controlled cell death in the development and maintenance of tissue homeostasis. There are two main apoptotic pathways: the intrinsic or mitochondrial-mediated pathway and the extrinsic or death receptor-mediated pathway Mitochondrial-mediated apoptosis is controlled by Bcl-2 family of proteins. The death receptor-mediated pathway is initiated by the ligation of cell death ligands with their death receptors. Caspase-3 is activated in both apoptotic pathways and plays central role in the execution-phase of cell apoptosis. Apoptosis is usually deregulated in cancer cells and this deregulation can contribute to uncontrollable proliferation and tumor growth. Opioids, especially morphine, are the most effective drugs available clinically for the management of severe pain associated with cancer. However, opioids can also affect proliferation, migration and apoptosis of tumor cells. This study was designed to test the effect of different opioids such as: morphine, endomorphin-2, and morphiceptin on some crucial apoptotic gene expression in MCF-7 breast adenocarcinoma cells.

Methods: The expression of studied genes was quantified using real-time RT-PCR

Results: All tested opioids increase the expression levels of pro-apoptotic genes, bax and caspase-3, and decrease anti-apoptotic gene bcl-2 expression.

Conclusion: The findings reported in this study may be useful in designing further experiments aimed at elucidating the role of the opioids in apoptosis machinery. The ability to modulate the life or death of a cell by opioids makes them potential targets for drug development.

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PS II – 2

Lack of agonist-induced desensitization of μ-opioid receptors located at nerve terminals

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Background and Aim: Agonist-induced desensitization of μ opioid receptors (MOPrs) is one of the leading mechanisms thought to underlie the development of tolerance to opioid drugs such as morphine and heroin. Although many studies have investigated desensitization of MOPrs located on cell bodies, few have examined MOPrs located at nerve terminals. In the ventral tegmental area (VTA), a brain region implicated in the rewarding properties of numerous drugs of abuse, MOPrs are located on GABAergic interneurones both somatodendritically (ie. on cell bodies and dendrites) and on nerve terminals that innervate dopaminergic neurones.
Methods: Using whole-cell patch-clamp electrophysiological methods, we have investigated agonist-induced desensitization of both populations of MOPrs in mouse VTA slices. Actions of MOPrs at nerve terminals were assessed by recording miniature and evoked inhibitory postsynaptic currents (IPSCs) from the postsynaptic dopaminergic neurones. Cell body MOPr activity was measured by recording G-protein activated inwardly rectifying potassium channel (GIRK) currents directly from GABAergic neurons.
Results: Both morphine (30 μM) and DAMGO (10 μM) inhibited the frequency of miniature IPSCs and decreased the amplitude of evoked IPSCs. These effects were sustained during 10-minute applications, even in conditions where there was no receptor reserve. In contrast, MOPrs at the cell bodies of GABAergic neurones rapidly desensitized; DAMGO (10 μM) induced a GIRK current that declined by approximately 50% during a 10-minute application.
Conclusions: In the mouse ventral tegmental area, MOPrs located at nerve terminals do not readily desensitize, or do so by different mechanisms to those located at cell bodies.

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PS II – 3

The effect of sodium ion on μ-opioid receptor G-protein activation

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Background and Aim: During functional [^{35}S]GTPγS binding assays, in which the G-protein activation is measured, the used buffer contains 100 mM NaCl solution, which probably decreases the agonist binding in a high extent, thus the agonist induced G-protein activation could also be highly inhibited. Our aim was to investigate agonist induced G-protein responses in the absence, and in the presence of different concentration of Na^+ in μ-opioid receptors (MOR) in rat brain membrane homogenates.
Methods: In our experiments we used functional [^{35}S]GTPγS binding assays to monitor G-protein activation. The MOR was activated by a highly selective [d-Ala^2,$_{NMe}$Phe^4,Gly^5-ol]enkephalin (DAMGO) syn-

thetic peptide. NaCl was added either only directly to the tubes in the absence or presence of DAMGO (10 μM), or either just in to the assay buffer.
Results: Sodium ion decreased the basal activity of G-protein in a concentration dependent manner. When the receptor was activated with DAMGO the highest stimulation was in 10 and 25 mM concentrations of NaCl. If the ion was added in the assay buffer the strongest stimulation was in the 100 mM concentration, and at the same time the affinity of DAMGO was the lowest.
Conclusion: We assume that the reason why the G-protein activation culminates in 100 mM NaCl concentration is because it is the nearest to the physiological NaCl concentration, which is 150 mM. Thus in the future it is expedient to carry out experiments in such concentration.

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PS II – 4

RGS2 and RGS4 proteins act as novel modulators of κ and δ opioid receptors signaling

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Background and Aim: Regulators of G protein Signaling (RGS) comprise a large multifunctional protein family that accelerate GTP hydrolysis of Gα subunits, thus modulating G protein coupled receptor (GPCR) signaling. RGS proteins also act as effector antagonists and serve as "platforms" where protein complexes can be formed [Willars , 2006]. We have previously demonstrated that RGS4 directly interacts with μ (μ-OR) and δ (δ-OR) opioid receptors to regulate their signaling [Georgoussi et al., 2006; Leontiadis et al. 2009]. To deduce whether selectivity in coupling between members of RGS proteins and opioid receptors exist we tested the ability of members of B/R4-RGS family to interact with κ opioid receptor (κ-OR).
Results: Pulldown experiments using GST fusion peptides encompassing the δ-third intracellular loop (δ-i3L) and the carboxyl terminal tails of all three opioid receptors subtypes (μ, δ, κ) indicated that RGS2 interacts within the δ-i3L and the C-terminal regions of δ-OR and κ-OR but not μ-OR. Co-immunoprecipitation studies indicated that RGS2 associates with κ-OR and δ-OR constitutively and retains upon agonist stimulation for both receptors. Moreover subcellular localization of both RGS proteins is altered upon receptor stimulation. RGS2 and RGS4 display a differential regulatory effect as assessed by a series of functional assays on κ-OR and δ-OR upon receptor activation.
Conclusion: Collectively, our results suggest that although κ-OR and δ-OR interact with the same subset of RGS proteins each of them affects signaling in a distinct manner.

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PS II – 5

δ-opioid receptor activation leads to neurite outgrowth and neuronal survival *via* a STAT5B-Gαi/o pathway

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Background and Aim: The opioid receptors participate in mechanisms controlling neural growth, differentiation and synaptic plasticity [Christie, 2008]. We have recently demonstrated that δ- and μ- opioid receptors (δ-OR and μ-OR) form multi-component signaling complexes, consisting of STAT5A/B, c-Src kinase and selective G protein subunits, leading to STAT5A/B phosphorylation [Georganta et al., 2010; Mazarakou and Georgoussi, 2005]. It seems, therefore, plausible to speculate that opioids receptors may modulate differentiation of neuronal cells involving members of the STAT family.
Result: To examine the effect of δ-OR activation on neuronal survival and neurite outgrowth, Neuro-2A cells were treated with various ligands and a) the number of live cells was visualized and counted under a microscope in the presence of trypan blue, or b) the length of the neurites was measured. Our results have shown a higher percentage of surviving cells in the presence of DSLET, an effect that was reversed by antagonist co-treatment or expression of a dominant negative STAT5B construct (DN-STAT5B). Similarly, agonist administration resulted to increased neurite outgrowth and this effect was blocked by pertussis toxin pre-treatment and the presence of DN-STAT5B.
Conclusion: Taken together, our findings demonstrate that δ-OR activation leads to neuronal cell survival and neurite outgrowth *via* a signaling pathway involving Gαi/o proteins and STAT5B.

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PS II – 6

A novel mechanism of signal transmission: "prototypical" interactions of neuropeptides with plasma membrane

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Background and Aim: Several peptides including penetratin and Tat are known to translocate across the plasma membrane. We here demonstrate that dynorphin A (DA) and big dynorphin (BD), consisting of dynorphins A and B, can translocate across the plasma membrane of live neuronal and non-neuronal cells, and form pores in this membrane.
Methods: Confocal fluorescence microscopy, fluorescence correlation spectroscopy and patch-clamp techniques.
Results: DA and BD were found to be able to penetrate across the cell membrane into neurons and non-neuronal cells. The peptide distribution was characterized by substantial cytoplasmic and plasma membrane labeling with minimal signal in the cell nucleus. The translocation potential of DA was comparable to that of transportan-10, a prototypical cell penetrating peptide. A central BD fragment, which retains all basic amino acid residues and dynorphin B did not enter

the cells. The patch-clamp experiments demonstrated that BD at low micromolar concentrations, and DA at higher concentrations induced transient increases in the cell membrane conductance in cultivated neurons and in non-neuronal cells. The BD-induced conductance increases were voltage-dependent; their frequency increased at the low negative potential inside the cell. Translocation and pore formation were not mediated *via* opioid receptors. The potential of dynorphins to penetrate into cells and to form pores correlated with their ability to induce non-opioid effects in animals including pathological pain.

Conclusions: Translocation across the plasma membrane and pore formation may represent a hitherto unknown mechanism by which dynorphins can signal information across the plasma membrane.

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SESSION III: **Opioids in pain**

PS III – 1

Activation of opioid receptors in injured nerves is required for efficient analgesia in neuropathic pain

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Background and Aim: Pain arising from peripheral nerve injuries is one of the most debilitating forms of chronic painful conditions, and it is considered to be weakly responsive to opioids. In this study we examined whether opioid delivery relative to the site of nerve injury is important for efficient peripheral analgesia.

Methods: Two and 14 days after chronic constriction injury (CCI) we examined the expression of opioid receptors by immunofluorescence and mechanical allodynia using von Frey test in mice. Opioid receptor agonists and antagonists were injected at the CCI site or into the paw (*ipl*) innervated by injured nerve.

Results: We found that the number of μ- and δ-receptor expressing neurons in dorsal root ganglia did not change after CCI. However, in injured nerves, the intesity of μ- and δ-receptor staining was enhanced proximally to ligatures. In hind paws innervated by damaged nerves, the number of μ- and δ-receptor expressing fibers was unchanged or decreased, respectively. μ-, δ- and κ-receptor selective agonists (DAMGO, DPDPE and U50,488H, respectively) injected at the CCI site or *ipl* produced dose-dependent analgesia. Interestingly, the potency and efficacy of opioids was substantially higher after their application at the CCI site than *ipl*. The analgesic effects of agonists were blocked by the respective selective receptor antagonists applied locally.

Conclusions: Our studies suggest that activation of opioid receptors directly at the site of nerve injury is critical for efficient peripheral analgesia in neuropathic conditions.

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No conflict of interest exists.

PS III – 2

The influence of minocycline on injury-induced changes in dynorphin and nociceptin expression and effects of KOP and NOP receptor ligands in a rat model of neuropathic pain

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Background and Aim: A role of neuropeptides in neuropathic pain development has been implicated; however, the neuroimmune interactions that are involved in the underlying mechanisms may be more important than previously thought. The aim of our study was to examine the effects of inhibition of glia activation by minocycline on injury-induced dynorphin and nociceptin changes and effects of KOP and NOP receptor ligands in a rat model of neuropathic pain.
Methods: The chronic constriction injury (CCI) of the rat sciatic nerve was performed. The opioid receptor ligands and minocycline were injected intrathecally or intraperitonealy. Two behavioral tests were conducted to measure allodynia (von Frey test) and hyperalgesia (cold plate test). For biochemical studies the RT-PCR was used. The experiments were carried out according to IASP rules (Zimmermann, 1983).
Results: The RT-PCR results indicated a strong upregulation of prodynorphin mRNA with no changes in the expression of pronociceptin in the spinal cord after chronic constriction injury (CCI). Changes in prodynorphin were parallel to higher expression of microglia marker C1q mRNA. In the DRG, a very strong upregulation of prodynorphin (1387%) and pronociceptin (122%) mRNAs was observed. Interestingly, preemptive and repeated intraperitoneal injection of minocycline inhibits the activation of C1q positive cells and upregulation of prodynorphin and pronociceptin in the DRG. We demonstrate that anti-allodynic effect of intrathecal administration of U50,488H (25–100 μg), but not dynorphin 0.15–15 μg) and nociceptin (0.05–5 μg) were significantly potentiated by preemptively and repeatedly injected minocycline.
Conclusions: We present evidence that glial inhibition not only diminishes neuropathic pain-related behavior, but also reduces the injury-enhanced expression of prodynorphin and nociceptin in the DRG. The behavioral studies underline that glia activation can be an important factor especially in kappa-opioid mediated analgesia.

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PS III – 3

The possible role of endomorphin 2 biosynthesis and CART peptide in the nociceptive information processing in rat spinal dorsal horn: pharmacology and immunohistochemistry

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Background and Aim: While demonstrating the possibility of de novo biosynthesis of endomorphin 2 in rat isolated L4,5 dorsal root ganglia from Tyr-Pro precursor in the presence of DPP4 inhibitor Ile-Pro-Ile, besides the heretic idea itself, two further features had to be considered. First, the biosynthesis took place at the external surface of plasma membrane. Second, while Tyr-Pro had to be provided exogenously, the other co-substrate must have been generated endogenously. Since similar endomorphin biosynthetic rules operate in rat spinal dorsal horn in carrageenan induced inflammation, we investigated these aspects as well.
Methods: Carrageenan-induced hyperalgesia was studied in male Wistar rats. Drugs were given mainly intrathecally, occasionally subcutaneously. Morphologically multiple immunostaining was combined with confocal microscopy or silver-intensified immunogold staining combined with electron microscopy.
Results: Inrathecally injected rCART(55-76) peptide, which carries the N-terminal Ile-Pro-Ile motif, exerted endomorphin 2- and opioid receptor-mediated antihyperalgesic effect. Using quadruple immunostaing in transverse sections of rat spinal cord at L4,5 level followed by confocal microscopy, we have shown in laminae I-IIo the neural co-localization of endomorphin 2, CART and substance P (possible Phe-Phe-Gly source) and contacts by NPY-containing profiles (possible Tyr-Pro source). Furthermore increased plasma membrane apposition with scattered intracellular staining of endomorphin 2 was found by electron microscopy in sections from plantarly carrageenan-, intrathecally Ile-Pro-Ile-treated rats.
Conclusions: These morphological findings do not prove either that endomorphin 2 biosynthesis takes place the way we have proposed, or it happens at the external plasma membrane surface, they are at least suggestive for these possibilities.

PS III – 4

Dose-dependent potentiation by the δ antagonist cha-TIPPpsi on the spinal antinociceptive effect of the μ agonist DAMGO in opioid-naive rats

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Background and Aim: The role of δ opioid receptors in analgesia and the development of opioid tolerance is not clear. We reported that the selective δ-antagonist TIPPpsi eliminated the tolerance to the intrathecal (it) analgesic (tail-flick) action of the selective μ agonist DAMGO in mice and rats. This potentiating

effect was observed in opioid-naive rats as well. We asked whether the potentiating effect of cha-TIPPpsi is dose-dependent.
Methods: MVD: Male NMRI mice (30–35 g) were used. The inhibitory effects of DAMGO and the selective delta agonist DPDPE were measured and the equilibrium constants of CTAP and cha-TIPPpsi were calculated. Tail-flick test: Male Wistar rats (150–200 g) were used. The effect of DAMGO was measured with and without cha-TIPPpsi given *it*. Antinociceptive ED50 values and their 95% confidence intervals were determined.
Results: In MVD both antagonists showed very high selectivities for μ and δ receptors, respectively. The Ke value of CTAP for the μ receptor was 20-fold less than that of cha-TIPPpsi for δ receptors. In tail-flick test 1 nmole/rat cha-TIPPpsi potentiated the antinociceptive effect of DAMGO but no potentiation was observed in the presence of 0.1 nmole/rat cha-TIPPpsi.
Conclusion: The potentiating effect of cha-TIPPpsi is dose-dependent. The required potentiating dose is surprisingly high. We hypothesize that cha-TIPPpsi does not bind to the δ receptor monomers but possibly to a μ-δ heterodimer. We suggest that the basal density of the μ-δ heterodimer in naive rats can be higher than in naive mice where no potentiation can be observed.

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PS III – 5

The antihyperalgesic and acute antinociceptive effects of intrathecally injected DAMGO, endomorphin 2 and the endomorphin 2 biosynthesis inducers ILE-PRO-ILE and vildagliptin in rats

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Background and Aim: We have shown previously in rat isolated L4,5 dorsal root ganglia that using depolarizing stimulus, the DPP4 inhibitor Ile-Pro-Ile (IPI) stimulated the de novo biosynthesis of endomorphin 2 from Tyr-Pro precursor. We have presumed that carrageenan-induced hindpaw inflammation creates conditions favouring endomorphin 2 generation in spinal dorsal horn. Indeed, intrathecally injected Ile-Pro-Ile and vildagliptin (VIL) exerted potent, endomorphin 2- and opioid receptor-mediated antihyperalgesic effect in the rat Randall-Selitto test whereas they had no analgesic action (at 30 nmol/rat) in the tail-flick test. We compare the antihyperalgesic and acute antinociceptive effects of DAMGO, endomorphin 2, Ile-Pro-Ile and vildagliptin
Methods: Radiant heat-induced tail flick was used to characterize acute nociception. Carrageenan-induced mechanical hyperalgesia was measured by the Randall-Selitto method. Direct intrathecal injection was used for drug delivery. Effects were tested 5-15-30-60 min after injection.
Results: Comparing the antihyperalgesic/acute antinociceptive potency ratios of *it* injected DAMGO, E2, IPI and VIL, the ratio for DAMGO was found 6.1-fold (5.9 pmol/rat ED50 in acute antinociception *vs* 0.96 pmol/rat antihyperalgesic ED50), for E2 15.5-fold (13.3 nmol/rat *vs* 0.86 nmol/rat) whereas the ratios for IPI and VIL were well over 20-fold (antihyperalgesic ED50s below 1.5 nmol/rat, no acute antinociception at 30 nmol/rat).
Coclusion: The data for DAMGO and EM2 match the tendencies reported by others previously and reasonable explanations can be given, whereas the case for IPI and VIL may be more complex.

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PS III – 6

Genetic ablation of δ opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes analgesia

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Background and Aim: Opioid receptors are major actors in pain control and are broadly distributed throughout the nervous system. A major challenge in pain research is the identification of key opioid receptor populations within nociceptive pathways, which control physiological and pathological pain. In particular, the respective contribution of peripheral versus central receptors remains unclear, and has not been addressed by genetic approaches.

Methods: To investigate the contribution of peripheral δ opioid receptors in pain control, we created conditional knockout mice where δ receptors are deleted specifically in peripheral Nav1.8-positive primary nociceptive neurons.

Results: Mutant mice showed normal pain responses to acute heat, mechanical and formalin stimuli. In contrast, mutant animals showed a remarkable increase of mechanical allodynia under both inflammatory pain induced by Complete Freund's Adjuvant (CFA) and neuropathic pain induced by partial sciatic nerve ligation (SNL). In these two models, heat hyperalgesia was virtually unchanged. SNC80, a δ agonist administered either systemically (CFA and SNL) or intra-paw (SNL), reduced thermal hyperalgesia and mechanical allodynia in control mice. However, these analgesic effects were absent in conditional mutant mice.

Conclusion: This study reveals the existence of δ opioid receptor-mediated mechanisms, which operate at the level of Nav1.8-positive nociceptive neurons. δ receptors in these neurons tonically inhibit mechanical hypersensitivity in both inflammatory and neuropathic pain, and are essential to mediate δ opioid analgesia under conditions of persistent pain. This δ receptor population represents a feasible therapeutic target to alleviate chronic pain while avoiding adverse central effects.

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PS III – 7

The *in vitro* and *in vivo* pharmacology of c-14-substituted agonist and antagonist opioids

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Background and Aim: Opioid agonists and antagonists are used to manage pain and as opioid antidote, respectively. Morphine-6-sulfate (M6S) has been reported to have analgesic effect similar to morphine-6-glucuronide and much higher than morphine after central administration. In vitro (vasa deferentia of mouse, MVD and rat, RVD) and *in vivo* characterization of 14-O-methoxy-morphine-6-sulfate (14-O-MeM6S) and naltrexone-14-O-sulfate (NTX-14-O-S). **Results:** In MVD, the IC50 (nM) was 18.74, 109.14 and 974.7 for 14-O-MeM6S, DAMGO (D-Ala2,N-Me-Phe4,Gly5-ol-enkephalin) and morphine, respectively. The dissociation constant (Ke) values of naltrexone (NTX) indicate the selectivity of 14-O-MeM6S for μ-opioid receptors (MOR). To assess the efficacy of the test compounds, we used the RVD, displaying low MOR pool and only drugs with higher efficacy inhibit RVD contractions. In RVD, 14-O-MeM6S and DAMGO but neither M6S nor morphine produced inhibitory effects. The NTX-14-O-S Ke value (nM) was 6.88 against DAMGO (MOR selective agonist), 77.47 against DADLE (selective δ-opioid receptor agonist) and 87.78 against EKC (selective κ-opioid receptor agonist). In rat tail-flick test 14-O-MeM6S was more potent than morphine and M6S. *Sc*/*icv* ratio was 12887 for 14-O-MeM6S and 177 for morphine. NTX-14-O-S reversed the antinociceptive action of *sc* morphine in rat tail-flick.
Conclusion: Presence of methoxy group at C14 of M6S increased the activity and the efficacy of M6S, whereas the sulfate group at C14 in NTX retain the selectivity but ameliorate the affinity for MOR. The higher *sc*/*icv* ratio for 14-O-MeM6S may indicate the limited access of the compound into the brain.

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PS III – 8

H-Dmt-DLys-Phe-Phe-OH, a tetrapeptide metabolite of the opioid-neurotensin hybrid pepide PK20, expresses high antinociception

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Background and Aim: The clinical treatment of various types of pain relies upon opioid analgesic, however most of them produce, in addition to the analgesic effect, several side effects such as development of dependence and addiction as well as sedation and dysphoria. One of the solutions to these problems are chimeric compounds in which opioid pharmacophore is hybridized with other type of synergically active antinociceptor. Neurotensin-induced antinociception is not mediated through the opioid system.

Recently, we presented a novel highly antinociceptive compound PK20, combining in one molecule both opioid and neurotensin analogue pharmacophores. The metabolic studies of PK20 indicated formation of quite stable N-terminal tetrapeptide, named PK20M which is also a novel endomorphine-2 analogue with the sequence Dmt-Dlys-Phe-Phe-NH2.
The aim of presented study was to evaluate a pharmacological profile of PK20M which was therefore exposed to both *in vitro* and *in vivo* studies.
Methods: The receptor binding data to opioid receptors (μ and δ) was performed using rat membrane homogenates. Whereas measurements of antinociception was carried out by intrathecally administration of PK20M into Wistar rats and using tail-flick test, where role of nociceptor agent was fulfilled by a light beam.
Results: PK20M, being N-terminal metabolite of the opioid-neurotensin chimera PK20, expresses a high dose- and time-dependent analgesic activity. Moreover, its central administration at a dose of 0.02 nM/rat induced antinociceptive response stronger than morphine, which concentration was 150-fold lower.
Conclusion: Opioid metabolite PK20M is characterized by a very high affinity to mu opioid receptor thus generating a significantly intensified analgesic effect.

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PS III – 9

Antinociceptive effect induced by combination of opioid and neurotensin pharmacophores and their hybrid peptide [Ile9]PK20

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Background and Aim: Pain signals transmission and their perception are regulated by large number of neuromediators and neuromodulators. Endogenous opioids are major regulator of pain signal. Neurotensin involvement in modulation of pain signal are much more complicated. Recently, we developed peptide PK20 which chimerized opioid and neurotensin pharmacophore analogues. PK20 expressed high and prolong analgesic effects. The construction of chimeras are new chemical molecule, structurally distinct from parent molecules. Therefore, hybridization of two active components raises the question to what extension of such hybridization cross influence on activity of separated active components. To perform such structure activity studies separated opioid and neurotensin pharmacophores as well as their chimera have been synthesized and comparison of antinociceptive effects mediated by them were done.
Methods: Synthesis of a mixture of opioid and neurotensin pharmacophores and their covalently hybridized peptide [Ile9]PK20 were developed manually using a standard procedure of solid-phase peptide synthesis (SPPS).
To measure the analgesic activity drugs were administered intrathecally at various doses into rats and antinocisponsive action was evaluated using the tail-flick.
Results: Both opioid-neurotensin chimera [Ile9]PK20 and a conjunction of its pharmacophores exert time- and dose-dependent analgesia, however administration of a hybrid peptide seems to induce antinociceptive response not as strong as in case of the mixture of opioid and neurotensin parts alone.
Conclusion: Presented results shows that analgesia mediated by [Ile9]PK20 probably resulted from inhibitory interactions between both its hybridized parts. Moreover, it may be concluded that there are different pathways involved in [Ile9]PK20's and combination of its pharmacophores' analgesic effect.

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PS III – 10

The antinociceptive effects of central versus peripheral μ-opioid agonists in a rat model of visceral pain

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Background and Aim: The involvement of peripheral μ-opioid receptors in the peripheral antinociception of the μ-opioid agonists D-Ala2, N-Me-Phe4, Gly5-ol-enkephalin (DAMGO) and morphine is well documented. However, the effects of local versus central administration of these agonists in the rat writhing test are scarce.
Method: 2% acetic acid was injected intraperitoneally (*ip*) in Wistar rats to induce visceral nociceptive responses. At a time, when these nociceptive responses became stable (after 50 min) DAMGO and morphine were injected *ip* or *icv* and their antinociceptive effects were investigated over the next 20 min.
Results: Both *ip* and *icv* DAMGO as well as morphine inhibited writhing in a dose dependent manner. The antinociceptive effects of DAMGO and morphine were more potent after *icv* than *ip* administration. Co-administration of the peripherally restricted opioid antagonist naloxone methiodide (QNX) significantly reversed the antinociceptive action of *ip* DAMGO and morphine. On the other side, *icv* injections of QNX partially antagonized the antinociceptive effects of *ip* morphine, but not of DAMGO. Finally, *ip* injections of opioid antagonist naloxone abolished completely the antinociceptive effects of morphine or DAMGO.
Conclusion: The antinociceptive effects of morphine on rat visceral nociceptive responses are based on the activation of both central and peripheral opioid receptors. However, the antinociceptive effects of DAMGO are based mostly on the peripheral opioid receptors. Interestingly, central antinociceptive effects of *icv* DAMGO and morphine were more pronounced than peripheral effects resulting from *ip* administration.

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PS III – 11

Sadowski mice as a model of blood brain barrier variability

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Background and Aim: Bidirectional selection of Swiss-Webster mice for High (HA) and low (LA) swim stress-induced analgesia caused substancial differences in endogenous pain inhibition mechanisms triggered by stress. The developed HA/LA strains we named "Sadowski mice" Years of searching for reason of differences between LA and HA strains have not resulted in final clear conclusion. We hypothesized that the differences in leaking of the blood-brain barrier (BBB) is main differences between the LA and HA of Sadowski mice strains.
Methods: Complementary methods of BBB effectiveness have been applied, including (a) electron microscopy analysis of morphological changes of BBB,

(b) *in vivo* studies of analgesic effectiveness low and high permeable opioid analgesics, (c) effect of mannitol ("BBB opener") on peripheral application of analgesics with low and high BBB permeability.
Results:
- The electron microscopy analysis of BBB of LA and HA indicated morphological changes mainly in HA strain;
- Peripheral application of compounds with high BBB permeabilities resulted in central analgesia similar for both, HA and LA strains;
- Peripheral application of compound with lower BBB permeabilities resulted in central analgesia higher for HA than LA strain;
- Intravenous application of mannitol strongly influence LA strain, whereas in HA mice the effect of mannitol was very small.
Conclusion: Our studies suggests that changes in blood-brain barrier permeability are one of the major factors of differences between LA and HA strains of Sadowski mice.

PS III – 12

Hippocampal transcriptome associated with stress-induced analgesia phenotype in mice – involvement of nts2 receptors

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Background and Aim: Stress decreases pain sensitivity in laboratory rodents, a phenomenon known as stress-induced analgesia (SIA). Pain perception, sensitivity to analgesics and responses to stressors, including SIA, strongly depend on genotype and, in part, on hippocampus. The present study examined basal gene expression in hippocampus in lines of mice bred for high (HA) and low (LA) swim SIA. The lines differ in opioid system and the opioid and non-opioid types of analgesia.
Methods: To characterize between-the-lines differences in genetic correlates of hippocampal mechanisms of swim SIA whole-genome expression microarrays were used.
Results: We found 1.5 fold or greater differences between the lines in expression of 205 genes in the hippocampus. In contrast, in hypothalamus and raphe nuclei the transcriptome profiling revealed only 19 and 9 differentially expressed genes in naive LA and HA animals. These data indicates that selective breeding affected many aspects of hippocampal neurons physiology, including metabolism, structural changes and cellular signaling. Differentially expressed genes involved in calcium signaling pathway, including Slc8a1, Slc8a2, Prkcc, and Ptk2b, were up-regulated in LA. In HA mice we found robust up-regulation of genes coding receptors for neurotensin (Ntsr2) and GABA (Gabard) or GC-box-binding transcription factors (Klf5).
Conclusion: Our data indicate that selection for a single behavioral trait, swim SIA, results in alterations in hippocampal gene expression networks underlying involvement of hippocampus in SIA. Moreover, significantly increased constitutive expression of Ntsr2 and Gabard in HA mice suggests the genetic basis of the non-opioid type of SIA in HA mice.

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SESSION IV: **Opioid and cannabinoid interaction**

PS IV – 1

κ-opioid and cannabinoid-1 receptors mediate anti-inflammatory and antinociceptive effect of salvinorin a on experimental colitis in mice

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Background and Aim: Salvinorin A (SA), the primary active compound in the plant Salvia divinorum, inhibits motility and epithelial ion transport in the mouse gastrointestinal tract in a κ-opioid (KOR)- and cannabinoid receptor (CBR)-dependent manner. Our aim was to investigate the effects of SA on experimental colitis in mice.

Methods: Two experimental mouse models were used throughout the study, TNBS-induced model of Crohn's disease and DSS-induced model of ulcerative colitis. The anti-inflammatory action of SA was evaluated based on macro- and microscopic damage scores, and myeloperoxidase (MPO) levels. Changes in KOR and CBR expression were analyzed by Western Blot. The antinociceptive effect of SA was characterized based on the behavioural response to *ic* administration of oil of mustard (OM).

Results: SA (*ip*, *po*) dose-dependently reduced colonic inflammation scores in TNBS- and DSS-treated mice. The anti-inflammatory effect of SA was blocked by KOR, but not CBR, antagonists. Interestingly, a significant increase in KOR protein levels induced by TNBS-treatment was normalized by *ip* SA. In addition, SA (*ip*, *ic*) significantly decreased the number of pain responses after *ic* OM in control and TNBS-treated mice. The antinociceptive action of SA was blocked by KOR and CB1 antagonists.

Conclusion: SA displays a potent anti-inflammatory effect on experimental colitis in mice, mediated through KOR. The antinociceptive action of SA is KOR- and CB1-dependent.

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PS IV – 2

Cannabidiol affects body weight gain and adipose tissue accumulation in rats

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Background and Aim: Endocannabinoid and opioid systems are tightly linked and play important roles in the regulation of food intake and energy balance. Cannabidiol (CBD) is a major non-psychotropic constituent of Cannabis and has ability to act *via* both cannabinoid and opioid receptors. Our previous studies revealed decreased body weight gain following repeated CBD administration in the rapidly growing rats weighting approximately 280 g at the start of study. Now, we evaluated the effects of repeated CBD administration on body weight gain, food intake and adipose tissue accumulation under standard (SD) and high fat (HFD) diet.

Methods: Adult male Wistar rats (n = 36) weighing approximately 400 g at the beginning of the experiment, fed with SD only or with access to HFD (60% kcal from fat, 10% kcal from sucrose) received CBD for 14 consecutive days (5 mg/kg/day, intraperitoneal injections). Body weight gain and intake of food and water were measured daily. Total intra-abdominal fat was assessed by a dissection method.

Results: In contrast to previous findings, CBD treatment increased body weight gain in rats fed SD, but no significant change in food or water intake was observed. CBD did not affect weight gain in rats fed HFD, but it lowered preference for HFD and decreased intra-abdominal fat accumulation.

Conclusions: The results suggest that CBD affects regulation of body weight, adipose tissue accumulation, and preference for HFD. CBD may produce bidirectional effects depending on age or the metabolic state of the animal.

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PS IV – 3

A prototype cannabinoid receptor 1 antagonist rimonabant decreases μ-opiod receptor G-protein activation in mice forebrain

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Background and Aim: Opioids, cannabinoids and their receptors are well known for their overlapping pharmacological properties. These two systems are in tight interactions partly achieving their effects by altering each others receptor activity *via* direct and/or indirect mechanisms. A prototype cannabinoid-CB(1) receptor antagonist SR141716 (Rimonabant) is known as anorectic, anti-obesity drug. We investigated whether acute, intraperitoneal (*ip*) treatment as well as direct application of Rimonabant to a forebrain homogenate

has an impact on μ-opioid receptor (MOR) activation. We also tested the direct binding ability of Rimonabant to MORs in CB(1) receptor knockout mice forebrain.
Methods: We have performed functional [35S]GTPγS binding assay to study the changes on MORs G-protein activation, and radioligand binding competition experiments to directly measure Rimonabant binding to MORs. For both functional and receptor binding assays, we have used [D-Ala2,NMePhe4,Gly5-ol]enkephalin (DAMGO), a synthetic peptide agonist ligand with excellent MOR specifity.
Results: Acute *ip* injection of Rimonabant caused significant decrease in MOR-mediated G-protein activation both in $CB(1)^{+/+}$ wild type and $CB(1)^{-/-}$ forebrain. Direct application of 1 microM Rimonabant also significantly decreased the efficacy of DAMGO in both types of forebrain tissues. In competition binding assays we haven't seen any direct effect of Rimonabant on MOR binding sites both in $CB(1)^{+/+}$ and in $CB(1)^{-/-}$ forebrain membrane fractions. Direct application of Rimonabant in competition binding assays had only effect DAMGO binding in quite large 100 μM concentration.
Conclusion: Based on our data we assume that Rimonabant causes attenuation on the level of MOR G-protein activation without binding directly to the MORs.

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PS IV – 4

Changes in the cannabinoid (CB1) receptor expression level and G-protein activation in the kainic acid model of epilepsy

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Background and Aim: It is known that exogenous cannabinoids, such as tetrahydrocannabinol, the major constituent of cannabis drugs, such as marijuana, have anticonvulsant activity. Recent studies have advanced our understanding of the endogenous cannabinoid system and renewed the interest in cannabinoids as potential pharmacological tools for treatment of epilepsy. Endogenous cannabinoid system is becoming rapidly activated after seizure activity but still little is known about the molecular mechanisms underlining the role of the cannabinoid system in epilepsy.
Method: In the present study we have evaluated the effects of the CB1 (cannabinoid receptor1) agonist, ACEA (N-(2-Chloroethyl)-5Z,-8Z,11Z,14Z-eicosatetraenamide) on G-protein signaling using the agonist-stimulated [35S]GTPγS binding assay in membranes of the control and kainic acid treated rat hippocampus and cortex.
Results: Our results showed that ACEA displayed high potency and efficacy in stimulating the G-proteins and when compared to control animals, significant enhancements were observed in tissues from the kainic acid treated animals. Gene expression levels of the (CB1) receptor and cannabinoid receptor interacting protein 1 CRIP1 were also measured by RT-PCR, and both CB1 and CRIP1 expressions were found to be elevated in kainic acid treated animals.
Conclusion: For the first time, our present investigation demonstrates the increase in CB1 receptor agonist mediated G-protein stimulation in hippocampus and cortex in epileptic rat brain. The hippocampus seems to be the most vulnerable structure in epilepsy, in light of the observation of enhanced potency and efficacy produced by the CB1 agonist ACEA.

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PS IV – 5

Involvement of the endocannabinoid system and nitric oxide pathway in opioid analgesia in a rat model of neuropathic pain

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Background and Aim: A role of nitric oxide (NO) in the mechanisms of pain generation and transmission throughout the central and peripheral nervous systems has been implicated. It has been earlier reported that L-arginine/NO pathway is involved in antinociceptive mechanisms induced by analgesics, opioids and cannabinoids. Endocannabinoid anandamide (AEA) has been suggested to attenuate NO activity through the CB1 receptor. It is known that neuropathic pain states alter AEA level in the spinal cord which in consequence may influence NO activity and nociceptive processes in neuropathic pain.

Methods: Male Wistar rats were implanted with intrathecal catheters and 5 days later chronic constriction injury (CCI) to the sciatic nerve in rats was performed. Using the von Frey and cold plate tests, we examined the influence of the selective inhibitor of neuronal nitric oxide synthase (nNOS), TRIM (500 μg) as well as fatty acid amide hydrolase (FAAH) inhibitor, URB597 on nociceptive threshold and the effects of their intrathecal coadministration with DAMGO and morphine on analgesia in neuropathic rats on day 7 after CCI. Additionally, we examined also the effect of TRIM on endomorphin-1-induced analgesia in CCI rats. The experiments were carried out according to IASP rules (Zimmermann, Pain 16; 1983).

Results: DAMGO and morphine administration leads to a dose-dependent increase in the von Frey and cold plate response latencies. Increased cannabinoid system activity following injection of 10 ug URB597 potentiate opioid effects in CCI-exposed rats. Similarly, the inhibition of nNOS activity by TRIM increased morphine and DAMGO-induced analgesia. However, the influence of nNOS inhibition on endomorphin-1-induced analgesia was much less pronounced in comparison with two drugs mentioned above. Generation of NO *via* activation of nNOS in a spinal cord of neuropathic rats was observed at both mRNA and protein level and display a tendency to decrease after URB597 and TRIM administration.

Conclusion: These results suggest that NO is involved in the nociceptive processes and that activation of the NO pathway may diminish the efficiency of opioid antinociception in the central nervous system. Upregulated spinal endocannabinoid level in CCI rats indicate that the increase in AEA level is analgesic in CCI rats and possibly inhibits pain also *via* NOS pathway. The above data supports the importance of the postulated endogenous way of nNOS inhibition by increased spinal AEA levels and will be a subject of a further study.

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SESSION V: **Opioids in addiction**

PS V – 1

Persistent brain region-specific upregulation of vasopressin (V1ar) and oxytocin receptors in chronic intermittent escalating dose morphine administration in mice

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Background and Aim: Arginine vasopressin (AVP) and Oxytocin (OT) are two important hypothalamic peptides playing a key role in stress processes. There is evidence implicating the oxytocin and vasopressin systems in opioid addiction. Two subtypes of vasopressin receptors have been identified within the brain (V1aR and V1bR) whereas one type of oxytocin receptor has been characterized. The present study aims to further investigate the role of these systems in different phases of opioid addiction.

Methods: Quantitative autoradiographic mapping of vasopressin ([^{3}H]-AVP) and oxytocin ([^{125}I]- OVTA) receptors was carried out in chronically morphine-treated (20–100 mg/kg, *ip*, twice daily, 8 days) C57BL/6 male mice, and in mice withdrawn for 1 day (acutely), or 7 days (chronically) from this paradigm. To discriminate between V1aR and V1bR, a selective V1bR antagonist (SSR149,514) was used for displacement studies.

Results: Chronic morphine administration significantly increased SSR149,514-resistant [^{3}H]-AVP binding in the piriform cortex, nucleus accumbens, lateral septum and amygdala. This upregulation persisted following acute and protracted withdrawal. No detectable SSR149,514-sensitive vasopressin binding sites were observed in the brain regions analysed. Chronic morphine treatment increased [^{125}I]-OVTA binding in the olfactory nuclei which persisted following acute withdrawal, while long-term morphine withdrawal induced an increase in the [^{125}I]-OVTA binding in the piriform cortex, septum, nucleus accumbens, amygdala, ventromedial hypothalamus and periventricular thalamus.

Conclusions: These results indicate a profound dysregulation of the vasopressin and oxytocin systems by chronic morphine administration which persists after long-term withdrawal, suggesting that these systems might play an important role in the mechanisms underlying opioid addiction and opioid craving after abstinence.

PS V – 2

CP154,526, a selective inhibitor of CRF 1 receptor, blocks the acquisition of opioid withdrawal-induced conditioned place aversion in mice

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Background and Aim: Conditioned place aversion (CPA) is a highly sensitive index of the aversive motivational consequences of withdrawal from a chronic state of opioid dependence. Corticotropin-releasing factor (CRF) system has been implicated in the negative affective symptoms of opioid withdrawal. The CRF signal is transmitted by two distinct receptors, CRF1 (CRF1R) and CRF2, located at multiple anatomical sites. The purpose of the present study was to evaluate the changes in tyrosine hydroxylase (TH) expression in the ventral tegmental area (VTA), one of the most important anatomical substrates for drug reward an aversion, and the role of CRF1R in the negative affective states of opioid withdrawal.
Methods: Male mice received chronic morphine at increasing doses or saline, were conditioned by naloxone and killed immediately after the test. CP154,526 was administered 30 min the naloxone dose. The number of TH-positive neurons was determined by immunohistochemistry.
Results: The injection of naloxone to mice pretreated with morphine+vehicle induced CPA versus the dependent group treated with CP-154526 (a selective CRF1R inhibitor) and versus the group pretreated with saline instead of morphine. In addition, present data showed no changes in the number of dopaminergic neurons in the VTA when we compared the four groups studied.
Conclusion: These results provide pharmacological evidence for the importance of CRF1R in the negative affective states of naloxone-precipitated withdrawal.

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PS V – 3

Glucocorticoid (GC) receptor antagonist mifepristone attenuates morphine withdrawal behaviors

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Background and Aim: Several studies have shown that glucocorticoids may contribute to the aversive symptoms of withdrawal from exposure to drugs of abuse. Previous data from our laboratory have shown that the hypothalamus-pituitary-adrenal (HPA) axis represent one of the main systems implicated in stress adaptations. Different systems have been identified and controversial data have been shown about their role mediating the somatic signs of withdrawal from drugs of abuse. The aim of this study was to investigate the effect of the GC receptor blockade on the somatic symptoms of morphine withdrawal.
Methods: Rats were made dependent on morphine by means of two morphine pellets implantation (*sc*). Six

days later, rats were injected with the specific GC receptor antagonist, mifepristone (25 or 50 mg/kg, *ip*). Morphine withdrawal was precipitated by naloxone (1 mg/kg, *sc*) and the following opiate abstinence signs were assessed during a period of 30 min: jumping, wet-dog shakes, paw tremor, sniffing, writhing, tremor, teeth chattering, piloerection, rinhorrhea and chromodacryorrhea. Rats were also weighed before and 60 min after naloxone injection.
Results: Pre-treatment with mifepristone significantly attenuated the incidence of morphine withdrawal signs, such as wet-dog shakes, sniffing, tremor, ptosis, and piloerection (25 and 50 mg/kg). Also teeth chattering and chromodacryorrhea (50 mg/kg).
Conclusions: These data might suggest a main role for glucocorticoids on the expression of morphine abstinence syndrome signs which is in agreement with previous studies that have involved CRF receptors, such as CRF1R and CRF2R, on opiate withdrawal.

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PS V – 4

Disposition of opiate medications by human placenta

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Background and Aim: Treatment of the pregnant opiate addict with methadone (M) or buprenorphine (BUP) improves maternal and neonatal outcomes but is associated with neonatal abstinence syndrome (NAS). The lack of correlation between opiate dose and incidence and severity of NAS prompted us to the following hypothesis; human placenta acts as a functional barrier that regulates maternal to fetal transfer of opiate medications. Accordingly, fetal dependence on the opiate should relate to its concentration in the fetal circulation. The aim of our investigations is to determine placental disposition of the two opiate medications.
Results: The hypothesis was validated by the identification of the following; (1) Placental transfer, maternal to fetal, of methadone is > BUP (dual perfusion of placental lobule). (2) The enzyme CYP 19/aromatase is responsible for the biotransformation of M and BUP to EDDP and norBUP, respectively (placental microsomes). (3) The activity of the enzyme increases with gestational age (term is higher than preterm placentas). (4) The efflux transporter P-gp extrudes M but not BUP. The uptake of M by inside-out-vesicle (IOV) preparations from placental apical membranes exhibited saturation kinetics and a Kt of 300 nM. (5) The expression of P-gp decreases with gestation. (6) Single nucleotide polymorphisms (SNPs) in the gene expressing P-gp affect its expression. (7) The activities of CYP 19 and P-gp vary widely between individuals.
Conclusions: Placental functions responsible for disposition of opiate medications include regulation of transfer from maternal to fetal circulation, its biotransformation and its efflux from the feto-placental unit.

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PS V – 5

Role of CRFR1 in the activation of catecholaminergic neurons from ventrolateral medulla during naloxone-induced morphine withdrawal

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Background and Aim: Recent evidence suggests that corticotropin-releasing factor (CRF) system is an important mediator in the opioid withdrawal state, contributing to the anxiogenesis and aversive symptoms. Catecholaminergic neurons of ventrolateral medulla (VLM) have been related to alterations of exploratory behavior and to an enhancement in stress hormones. In this study we examined the impact of CRF1 receptor (CRF1R) deficiency linked to the expression of tyrosine hydroxylase (TH), during naloxone-induced morphine withdrawal in knockout and wild-type mice. For this purpose, animals took part of a conditioned place aversion (CPA) paradigm, as a measure of the aversive effect of morphine withdrawal.
Methods: Animals were made dependent on morphine and confined to a selected room after naloxone injection.
Results: A double-labeled immunohistochemistry of phosphorylated TH at serine 31 (Ser31 TH) with c-Fos, a stressor marker, revealed that the number of Ser31 TH positive neurons that coexpress c-Fos in VLM is lower in the CRF1R-deficient mice versus the wild-type group, after the conditioned place aversion paradigm. In addition, the number of c-Fos positive cells decreased in the knockout mice.
Conclusion: Our results suggest that CRF1R is partially involved in the decreased anxiety/stress behaviour during morphine withdrawal.

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PS V – 6

δFosB expression in the brain stress system from adrenalectomized rats during morphine dependence

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Background and Aim: δFosB is one transcription factor of the Fos family. Because of its long half-life, it persists for long periods after the cessation of drug use. As a result, it gradually accumulates, suggesting that δFosB could represent a mechanism by which drugs of abuse produce lasting changes in gene expression pattern long after the drug is withdrawn. On the other hand, it has been proposed that glucocorticoids may facilitate acquisition, maintenance and relapse of drug self administration. This study was designed to evaluate the possible modifications in δFosB expression in the brain stress system during morphine dependence in adrenalectomized rats.
Methods: Male Sprague-Dawley rats underwent sham adrenalectomized (SHAM) or bilateral adrenalectomized (ADX) and were rendered dependent

on morphine. Ten days after the surgery, rats were anesthetized and transcardially perfused. Using an immunohistochemical approach, FosB/δFosB immunoreactivity was determined in the brain stress system.
Results: δFosB was induced after chronic morphine administration in the hypothalamic paraventricular nucleus (PVN), the shell of the nucleus accumbens (NAc-shell), bed nucleus of the stria terminalis (BNST), central amygdala (CeA) and nucleus tractus solitarius (NTS) in both, SHAM and ADX rats. Nevertheless, this enhancement in δFosB expression was markedly reduced in the NAc-shell and in the CeA from ADX rats.
Conclusion: These data indicate that glucocorticoids may regulate at some extent the induction of δFosB in the brain stress system and suggest that glucocorticoids would have a role in the alterations in gene expression during opiate dependence.

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PS V – 7

Morphine withdrawal induces the activation of the orexinergic neurons in the rat lateral hypothalamus

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Background and Aim: The addiction process involves the activation of the brain stress system, producing the negative emotional state that becomes the powerful motivation for drug-seeking associated with compulsive use. Orexins are neuropeptides synthesized exclusively in lateral hypothalamic neurons (LH) that have been found to mediate some effects of stress on addiction behavior. The aim of the present study was to evaluate the possible activation of the orexinergic neurons during morphine dependence and withdrawal.
Methods: Rats were made dependent on morphine by subcutaneous implantation of two 75 mg morphine pellets under light ether anaesthesia. Control rats received placebo pellets containing lactose instead of morphine. On day 7, rats were given saline or naloxone (1 mg/kg) and killed by perfusion or decapitation 30, 60, 90 and 120 min later.
Results: The effects of morphine withdrawal on orexin-A (OX-A) neurons activity were determined by RT-PCR and inmunohistochemistry techniques. Our results show that naloxone-precipitated morphine withdrawal induces an increase of OX-A synthesis (as estimated by OX-A mRNA levels), which was not accompanied by modification in the number of orexinergic neurons in any of the three areas of the LH: DMH (dorsomedial hypothalamus), PFA (perifornical area) and LLH (lateral LH). However, we found an enhancement in the number of orexinergic neurons expressing c-Fos during morphine withdrawal.
Conclusions: These results indicate that OX-A neurons are activated during naloxone-induced morphine withdrawal and suggest that orexin projections to the brain stress system could play a role in morphine dependence.

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PS V – 8

Transdermal delivery of a buprenorphine/naltrexone combination for the treatment of polydrug abuse

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Background and Aim: There is evidence that a buprenorphine/naltrexone combination may be effective as a relapse prevention not only for opioid abuse, but for polydrug abuse, yet little work has been carried out to determine the optimum ratio of the two drugs. A significant problem with this treatment strategy arises from the fact that buprenorphine and naltrexone need to be administered by different routes. However, both buprenorphine and naltrexone can be delivered transdermally using iontophoresis, and we are currently using this technique to develop a buprenorphine/naltrexone combination therapy that is effective in reducing relapse to polydrug abuse.
Method: Using conditioned place preference (CPP) in male Sprague-Dawley rats, the rewarding properties of buprenorphine and naltrexone combinations were assessed. The ability of buprenorphine/naltrexone to inhibit drug-primed reinstatement of morphine-induced CPP was then tested.
Results: Buprenorphine alone (0.3 mg/kg) was rewarding, whereas a buprenorphine to naltrexone ratio of 1:10 (0.3 and 3 mg/kg) was aversive. However, a ratio of 1:3 (0.3 and 1 mg/kg) was neither rewarding nor aversive. A morphine priming dose of 2.5 mg/kg reinstated morphine CPP (animals were trained to demonstrate CPP with 10 mg/kg morphine followed by extinction training), an effect that was inhibited by prior administration of buprenorphine/naltrexone (0.3 and 1 mg/kg respectively). All drugs were administered *ip*
Conclusions: We have demonstrated a combination of buprenorphine/naltrexone with no rewarding or aversive effects that appears to inhibit reinstatement in an animal model of drug-seeking behavior. Ongoing work is designed to further test the ability of buprenorphine/naltrexone to prevent CPP reinstatement, and to optimise conditions for their transdermal delivery.

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PS V – 9

Role of the corticotropin-releasing factor receptor-1 in the conditioned preference place induced by morphine in mice

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Background and Aim: Corticotropin-releasing factor (CRF) plays a major role in regulating behavioural and hormonal responses to stress in animal models. It has been proposed that CRF system contribute in an important way at compulsive drug use. The purpose of the present work is to evaluate the ability of the selective CRF1 receptor (CRF1R) antagonist, CP154,526, in the conditioned preference place (CPP) induced by morphine in male mice.
Methods: The rewarding effects of morphine were evaluated using the CPP paradigm. We gave a dose of CRF1R antagonist 30 minutes before morphine or sa-

line. Animals were weighted every day during the experiment. The CPP score was calculated for each mouse as the difference between time spent in the drug-paired compartment during the postconditioning and the preconditioning phase.
Results: Mice treated with morphine showed a significantly lower weight gain than animals receiving saline injection, the administration of CRF1R antagonist didn't modify the weight gained observed during morphine-dependence. Moreover, a significant rewarding effect of morphine was observed in the place conditioning paradigm. The administration of CP154,526 prevent the CPP acquisition in animals treated chronically with morphine compared to animals which received vehicle.
Conclusion: Data suggest that CRF1R is firmly connected to the conditioned preference place induced by morphine in mice. In contrast, the administration of CP154,526 didn't change the impact of morphine on the weight gain observed.

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PS V – 10

Cell-type specific regulation of SGK1 isoforms by morphine and dexamethasone

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Background and Aim: Our previous research indicated that morphine regulates the expression of a number of genes in the mouse brain (Korostynski et al. 2007). Interestingly, we identified a large group of genes which expression is regulated by glucocorticoid receptor (GR). Serum and glucocorticoid-regulated kinase 1 (SGK1) belongs to this category and is one of the most strongly upregulated transcripts in the mouse striatum after single and chronic morphine administration. Sgk1 is regulated by a variety of factors, and was recently suggested to play important role in the process of learning and memory. Since there are few transcriptional isoforms of Sgk1 gene, we aimed to investigate 1) the abundance of individual isoforms in different time points after single administration of morphine and other drugs of abuse, 2) their cell-type specific regulation by morphine and GR agonist – dexamethasone.
Methods: We used cell-type specific culture systems and variant-specific probes in qRT-PCR and in situ hybridization of brain slices.
Results: We found that:
– sgk1 and sgk1.2 were upregulated by all drugs of abuse *in vivo* with the strongest effect 2 h after morphine and heroin administration;
– sgk1 and sgk1.2 were expressed in glia-specific cell cultures and were upregulated by dexamethasone *in vitro*;
– sgk1.1 was expressed almost exclusively in neurons, but its level remained constant regardless of the treatment both *in vitro* and *in vivo*.
Conclusions: In summary, we describe sgk1 and sgk1.2 as glia-specific transcripts regulated by drugs of abuse. The GR signaling pathway is the most probable candidate to be responsible for the reported changes.

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PS V – 11

Expression regulation of opioid propeptides and dopamine receptors in the mesostriatal system by chronic intake of palatable foods

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Background and Aim: Chronic (self-)administration of addictive drugs typically up-regulates the prodynorphin (PDYN) gene expression in the striatum or nucleus accumbens. Drug effects on other genes in the mesostriatal system, including proenkephalin (PENK) and dopamine receptors, have also been characterized. The aim of our study was to determine if prolonged intake of highly palatable foods, which may produce addiction-like behaviours, affects the mesostriatal gene expression in the same manner as drugs of abuse.
Methods: Two models of chronic intake of palatable foods by C57BL/6 mice were used: i) instrumental self-administration of cocoa-flavoured pellets (daily 1-h sessions for 20 days) and ii) diet-induced obesity (DIO) model, i.e. constant free-choice access to a chocolate-based diet for 10 weeks. mRNA levels of PDYN, PENK and the dopamine receptors D1 and D2 (D1R, D2R) were assessed in the mesostriatal system by in situ hybridization.
Results: The PDYN mRNA levels were down-regulated in the striatum and nucleus accumbens in the instrumental model, and in the central amygdala of DIO mice. The D2R transcript was down-regulated in the nucleus accumbens both in the instrumental self-administration and DIO model. Expression of PENK and D1R remained unchanged in both models.
Conclusions: Neuroadaptations in the mesostriatal system produced by chronic intake of palatable foods seem distinct from those produced by drugs of abuse. Whereas tasty food self-administration fails to up-regulate the PDYN gene (unlike addictive substances), its characteristic effect seems to be down-regulation of the D2R in the nucleus accumbens, particularly in the shell. This suggests that drug and food addictions involve different neuronal mechanisms.

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Opioid peptides

PS VI – 1

Naloxone influences the morphine-induced enhancement of plasma β-endorphin in stress-resistant pigs

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Background and Aim: The experiments were performed on 18 chronically catheterised pigs of commercial line 990 divided in DNA diagnostic assay (PCR) for mutation of the ryanodine receptor gene (RYR1) into 3 groups: dominant homozygous (NN) and heterozygous (Nn) – stress resistant, and recessive homozygous (nn) – stress susceptible. Plasma concentration of β-endorphin (BEND) and cortisol (COR) (RIA) were measured during 8 hours after a single intravenous dose of 1 mg/kg of morphine (MF) or 1 mg/kg of naloxone (NX) or MF pretreated with NX (MF+NX).

Results: The highest concentrations of BEND after MF were observed in NN and Nn pigs with maximal effect at 15 min (NN-190.38 ± 10.33, $p < 0.01$; Nn – 229.38 ± 7.89 pg/ml, $p < 0.001$, in comparison with the baseline and NX-treated pigs). The MF-induced elevation of BEND concentration was significantly ($p < 0.001$) decreased by NX in NN and Nn genotypes (at 15 min: NN – by 51.4%; Nn – by 59.6%). In nn pigs injection of MF+NX did not evoke significant changes in BEND concentration. The increase in plasma COR concentration after MF was observed in all genotypes with maximal effect at 180 min (NN – 189.03 ± 7.63; Nn – 137.28 ± 8.26; nn – 145.09 ± 7.56 ng/ml). These MF-induced effects were significantly ($p < 0.01$) decreased by NX in NN and Nn pigs (by 47.3 and 50.0%, respectively).

Conclusion: The obtained results suggest that morphine-induced increase in plasma BEND concentration concerns phenotypically "stress resistant" pigs (NN and Nn) only. It suggests that these effects may depend not only on the opioid receptor system (NX-sensitive), but also on genetically based differences in the release of opioids in RYR1 genotype pigs.

PS VI – 2

Autocrine regulation of human sperm motility by met-enkephalin

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Bakground and Aim: Sperm motility appears to be essential for natural reproduction and is an important feature and currently the most reliable predictor of male factor infertility. Enkephalins are opioid peptides present in human semen which are involved in the regulation of sperm cell function, but the action of these bioactive peptides is not completely understood. Although spermatogenic cells are a major site of precursor RNA expression, proenkephalin (PENK, protein precursor of met-enkephalin) is not efficiently translated in mouse testis. In spite of that, some of PENK-derived peptides are stored in the mature spermatozoa but the role of its products is not clearly in mature spermatozoa. Therefore, the aim of this study was to study the presence of PENK and met-enkephalin in human spermatozoa and to clarify the effects of endogenous met-enkephalin on sperm motility.
Methods: The expression and localization of PENK and met-enkephalin was analyzed by RT-PCR and inmunofluorescence techniques. Met-enkephalin secretion was analyzed by flow cytometry. The motility analysis was conducted by computer-assisted sperm analysis (SCA)
Results: We found transcript of PENK in mature spermatozoa and its protein as well as met-enkephalin were present in mature spermatozoa its inmunoreactivity decreased during the time. The inhibition of enkephalin metabolism increased the sperm motility, being this effect reversed by naloxone.
Conclusion: The present study demonstrates a new possible endogenous mediator of sperm motility, because we found that human ejaculated spermatozoa are able to secrete met-enkephalin and improve sperm motility. The autocrine regulation of sperm motility by enkephalins opens a new area of study in male fertility.

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Nigral dynorphin peptide levels are associated with L-DOPA-induced dyskinesia

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Background and Aim: Striatal prodynorphin mRNA levels correlate well with the severity of L-DOPA-induced dyskinesia in animal models of Parkinson's disease (PD), but the actual neuroactive opioid peptides in the target basal ganglia output structure substantia nigra have not yet been determined.

Methods: Here, we use MALDI-TOF Imaging mass spectrometry (IMS) for the topographical elucidation of neuropeptides with focus on opioid peptides and their changing concentrations in the substantia nigra in an experimental model of PD and L-DOPA-induced dyskinesia. Endogenous dynorphin peptides

were identified by Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry.
Results: Dyskinesia rating revealed two separate groups of animals; one high-dyskinetic group and one low-dyskinetic group. Masses corresponding to prodynorphin (PDYN) derived peptides were of focus, there among dynorphin A (1–17), dynorphin A (1–8), alpha-neoendorphin (aNeo), dynorphin B (DynB) and their metabolites. DynB and aNeo were significantly upregulated in the lesioned side of the brain in the high-dyskinetic group (DynB; 75% and aNeo; 54%). Furthermore the peak intensity of DynB and aNeo were positively correlated to the dyskinesia score (DynB; $p < 0.05$, $R = 0.72$, aNeo; $p < 0.01$, $R = 0.82$). The metabolite Leu-Enk-Arg displayed a similar pattern as DynB and aNeo. Multiplexing DynB and Leu-Enk-Arg revealed areas of low DynB peak intensity and higher Leu-Enk-Arg peak intensity, suggesting that dynorphin B has been released and metabolized to Leu-Enk-Arg.
Conclusion: The MALDI IMS data from this study provides information in an unbiased way that would be difficult to find using other methods without prior hypothesis.

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Opioid-like immunoreactivity in the mamillary body of the guinea pig in fetal and postnatal stages of development

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Background and Aim: The mamillary body (MB) is associated with memory, learning and various physiological processes. Our previous studies have suggested that two systems of opioid precursors (proenkephalin and prodynorphin genes) are expressed in that region in the pig. The aim of this study was to examine immunoreactivity (ir) of products of these precursors in the development of the mamillary body in the guinea pig.
Methods: Brains from fetal stages (E30, E60), newborn (P0) and adult (PD) animals were fixed in 4% paraformaldehyde in phosphate buffer and then cryoprotected. Frozen sections were immunostained with the fluorescent technique using the primary antibodies against leu-enkephalin (LENK), α-neo-endorphin (END) and dynorphin A (DYN). The specific secondary antibodies were conjugated with fluorochromes.
Results: In E30 stage there was a low level of LENK-ir structures, observed in the differentiating perimamillary area as single, fine fibres, and in the mamillary area as dots in weak immunoreactive neuropil. In stage E60 LENK-ir fibres were located in whole area of the MB, especially in the most ventral region of it, close to the mamillary capsule. A level of density of LENK-ir structures in P0 stage was higher than in the prenatal stages. In E60, P0 and PD stages the patterns of DYN and END immunoreactivity were very similar. Single long fibres were located within the MB, whereas fibres of various morphology in the perimamillary region.
Conclusions: It seems that leu-enkephalin system innervates the mamillary body as the first from the studied opioids in fetal period and probably acts in non-opioid way.

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Opioids involvement in the regulation of metabolic syndrome

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Background and Aim: Endogenous opioid peptides are involved into many different metabolic processes , also in the regulation of blood pressure and cardiovascular diseases as well as inflammation. It is suggested that some of the peripheral and central pathophysiological symptoms associated with diabetes may be attributed, in part, to altered activity of enkephalinergic systems. The aim of the study was to measure the changes of the mRNA proenkephalin expression and Met-enkephalin concentration in the brain of diabetic piglets.
Results: Diabetes, DM I and DM II, were induced in the piglets by the streptozotocin and glucocorticoids, respectively. Expressions of mRNA proenkephalin in the hypothalamus and pituitary were measured by in situ hybridization, the concentration of native and cryptic Met-enkephalin was estimated by RIA method. As a proof of metabolic syndrome the visfatin and resistin mRNA expressions in the epicardium adipose tissue (EAT) were tested by Q-PCR method. Both, DM I and DM II significantly increased the concentration of native and cryptic Met-enkephalin in the hypothalamus and pituitary ($p < 0.01$). The expression of proghrelin mRNA in the brain fragments was changed by diabetes ($p < 0.01$). The resistin mRNA expression in the EAT was increased by 240% in the DM I and by 196% in the DM II ($p < 0.01$). In contrast, the visfatin mRNA expression was decreased from 1.00 to 0.04 (DM I) and from 1.00 to 0.316 RQ/18sRNA (DM II).
Conclusion: Thus, the obtained results clearly showed the involvement of opioids in the regulation of metabolic syndrome in the diabetic piglets.

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Investigation on DNA methylation status of opioid peptides promoters in PBMCs of subjects with bipolar disorder

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Background and Aim: The pathophysiology of mental illnesses is poorly understood. Many evidences support the hypothesis, in addition to dopamine and serotonin, of a role for the endogenous opioid peptides, in particolar dynorphin and nociceptin, whose peptides and precursors levels have already been found to be affected in psychiatric illness. We investigated, in subjects with bipolar disorders (BD), dysregulation of DNA methylation, since it has been proposed that altered expression of multiple mRNAs, in psychotic subjects may be due to epigenetic mechanisms. Previous studies suggested that peripheral blood is a useful peripheral marker and model of epigenetic gene regulation in the brain.

Methods: DNA was isolated from blood of patients diagnosed with BD either type I or II (according to DSM-IV criteria), and from healthy controls and bisulfite conversion was performed. Real-Time Methylation Specific PCR (MSP) was used for the quantification of the methylated promoters in all samples under study. The percentage of methylation was calculated by the 2-DDCt method, where DDCt = (Ct,Target – Ct,Myod)sample – (Ct,Target – Ct,Myod) fully methylated DNA and multiplying by 100 where Myod is the internal reference gene to control for input DNA.

Results: An increase in DNA methylation of dynorphin promoter region was observed in BD II patients (but not in BD I) compared to controls whereas no significant differences were found for DNA methylation of nociceptin promoter.

Conclusion: Our preliminary findings, showing selective changes in dynorphin regulation by epigenetic mechanisms, provide new insight in the possible involvement of dynorphin in mediating susceptibility to neuropsychiatric diseases.

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L-DOPA-induced dyskinesia is associated with regional increase of striatal dynorphin peptides as elucidated by imaging mass spectrometry

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Background and Aim: A strong association between L-DOPA-induced dyskinesia (LID) and elevated striatal prodynorphin mRNA levels has been established in both patients and in animal models of Parkinson's disease (PD), but to date the endogenous prodynorphin peptide products have not been determined.

Methods: Here, matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) was used for characterization, localization, and relative quantification of striatal neuropeptides in a rat model of LID in PD.

Results: IMS analysis revealed elevated levels of dynorphin B, α-neoendorphin (1-12, 2-12), substance P (SP), and PEnk (220–229) in the dorsolateral striatum of high dyskinetic animals compared to low dyskinetic and lesion-only control rats. Interestingly, only the peak intensities of the prodynorphin-derived peptides, dynorphin B and α-neoendorphin, were strongly and positively correlated with LID severity. In addition, the peak intensities of a putative metabolite lacking the N-terminal tyrosine of α-neoendorphin correlated positively with dyskinesia severity.

Conclusions: This first MALDI IMS-based study on neuropeptide analysis in experimental PD and LID, and the unique methodological approach facilitated comprehensive investigation of LID-associated prodynorphin-derived peptide products. We find that the dynorphins associated with LID are not mainly those with high affinity to κ opioid receptors, but consists of shorter dynorphins, mainly dynorphin B and α-neoendorphin, which are known to bind and activate also μ and δ opioid receptors. Des-tyrosine dynorphins display reduced opioid receptor binding and this points to possible compensatory non-opioid mediated changes in the striatum. Des-tyrosine dynorphins can only be detected by mass spectrometric method as no antibodies are currently available.

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